

Meta-Analyses of Microarrays of *Arabidopsis asymmetric leaves1 (as1)*, *as2* and Their Modifying Mutants Reveal a Critical Role for the ETT Pathway in Stabilization of Adaxial–Abaxial Patterning and Cell Division During Leaf Development

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It is necessary to use algorithms to analyze gene expression data from DNA microarrays, such as in clustering and machine learning. Previously, we developed the knowledge-based fuzzy adaptive resonance theory (KB-FuzzyART), a clustering algorithm suitable for analyzing gene expression data, to find clues for identifying gene networks. Leaf primordia form around the shoot apical meristem (SAM), which consists of indeterminate stem cells. Upon initiation of leaf development, adaxial–abaxial patterning is crucial for lateral expansion, via cellular proliferation, and the formation of flat symmetric leaves. Many regulatory genes that specify such patterning have been identified. Analysis by the KB-FuzzyART and subsequent molecular and genetic analyses previously showed that *ASYMMETRIC LEAVES1 (AS1)* and *AS2* repress the expression of some abaxial-determinant genes, such as *AUXIN RESPONSE FACTOR3 (ARF3)/ETTIN (ETT)* and *ARF4*, which are responsible for defects in leaf adaxial–abaxial polarity in *as1* and *as2*. In the present study, genetic analysis revealed that *ARF3/ETT* and *ARF4* were regulated by modifier genes, *BOBBER1 (BOB1)* and *ELONGATA3 (ELO3)*, together with *AS1–AS2*. We analyzed expression arrays with *as2 elo3* and *as2 bob1*, and extracted genes downstream of *ARF3/ETT* by using KB-FuzzyART and molecular analyses. The results showed that expression of *Kip-related protein (KRP)* (for inhibitors of cyclin-dependent protein kinases) and *Isopentenyltransferase (IPT)* (for

biosynthesis of cytokinin) genes were controlled by *AS1–AS2* through *ARF3/ETT* and *ARF4* functions, which suggests that the *AS1–AS2–ETT* pathway plays a critical role in controlling the cell division cycle and the biosynthesis of cytokinin around SAM to stabilize leaf development in *Arabidopsis thaliana*.

Keywords: *ASYMMETRIC LEAVES2 (AS2)* • *AUXIN RESPONSE FACTOR3/ETTIN* • CDK inhibitors • Cytokinin • Shoot apical meristem.

Abbreviations: CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; GCOS, Gene Chip Operating Software; KB-FuzzyART, knowledge-based fuzzy adaptive resonance theory; qRT–PCR, quantitative real-time reverse transcription–PCR; SAM, shoot apical meristem; ta-siRNA, trans-acting short interfering RNA; TSA, trichostatin A.

Introduction

Over the past several years, rapid advances in DNA microarray technologies, including a tiling array procedure, have made it possible to measure the expression levels of thousands of genes simultaneously and under different conditions (Yoshimura et al. 2011). These volumes of gene expression data can be used to reveal the mechanisms that drive life. To analyze this large quantity of data, however, requires the use of algorithms,

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as used in clustering and machine learning. We previously developed knowledge-based fuzzy adaptive resonance theory (KB-FuzzyART), which facilitates clustering analysis on the basis of a home-made list including a limited number of genes of interest, and applied the analysis of gene expression data to identify genetic networks that might be responsible for a physiological process (Takahashi et al. 2008).

In general, the objective of a statistical or informatical analysis is the enrichment of important information. In particular, we have selected the KB-FuzzyART clustering method in the present study. This method can enable us not only to enrich important information, but also to separate information into that which is known or unknown. We updated our previously used gene list (Gene-list-1) of *Arabidopsis thaliana* to make a new gene list (Gene-list-2). Using Gene-list-2, we applied KB-FuzzyART to two series of microarray data, to identify the genes downstream of *ASYMMETRIC LEAVES1* (AS1) and *ASYMMETRIC LEAVES2* (AS2) of *A. thaliana*, both of which are involved in leaf development (Byrne et al. 2000, Iwakawa et al. 2002, Semiarti et al. 2001).

Leaf primordia are formed at the periphery of the shoot apical meristem (SAM), which contains aggregates of indeterminate stem cells. Homeostasis of SAM was recently shown to be controlled by multiple receptor protein kinases as well as the *SHOOT-MERISTEMLESS* gene (Takano et al. 2010, Betsuyaku et al. 2011, Uchida et al. 2013). As each leaf grows, its morphology becomes established along three axes, the proximal–distal, adaxial–abaxial and medial–lateral axes. Adaxial–abaxial patterning at the initial stage, occurring in regions adjacent to the SAM, is critical for the lateral expansion of the lamina along the medial–lateral axis for formation of flat symmetric leaves (Waites and Hudson 1995, Byrne et al. 2000, Semiarti et al. 2001, Tsukaya 2006, Iwakawa et al. 2007, Bowman and Floyd 2008, Szakonyi et al. 2010, Moon and Hake 2011, Yamaguchi et al. 2012).

In *A. thaliana*, many genes for putative transcription factors have been identified as regulators of leaf development. Three members of the family of class III homeodomain-leucine zipper genes, such as *PHABULOSA* (PHB), specify the adaxial identity of lateral organs (McConnell and Barton 1998, McConnell et al. 2001, Emery et al. 2003, Bao et al. 2004, Mallory et al. 2004). Members of the *KANADI* (KAN) and *FILAMENTOUS FLOWER* (FIL)/*YABBY* (YAB) gene families have been identified as abaxial determinants (Bowman and Smyth 1999, Eshed et al. 1999, Sawa et al. 1999, Siegfried et al. 1999, Eshed et al. 2001, Kerstetter et al. 2001, Bowman and Floyd 2008, Goldshmidt et al. 2008, Sarojam et al. 2010). The other components determining abaxial cell identity include the *AUXIN RESPONSE FACTOR3* (ARF3)/*ETTIN* (ETT) and *AUXIN RESPONSE FACTOR4* (ARF4) genes (Sessions and Zambryski 1995, Kim et al. 1997, Sessions et al. 1997, Ulmasov et al. 1997, Pekker et al. 2005, Wu et al. 2008, Kelley et al. 2012). The results of investigations of these previously identified transcription factors support the hypothesis that the specification of adaxial–abaxial polarity might be tightly coupled with lateral expansion,

because inhibition of the development of either the adaxial or abaxial domain causes the appearance of filamentous leaves with the surface characteristics of either domain (Waites and Hudson 1995). Recently, it has been reported that succinic semialdehyde dehydrogenase is involved in leaf patterning (Toyokura et al. 2011). *AUXIN RESISTANT3* also has a role in the formation of flat leaves of *A. thaliana* (Pérez-Pérez et al. 2010). Nevertheless, information on gene networks that might control these regulators remains elusive.

AS1 and AS2 are key regulators of the formation of flat symmetric leaves. AS1 and AS2 encode nuclear proteins and form a complex (referred to as AS1–AS2 in this report; Xu et al. 2003, Yang et al. 2008, Luo et al. 2012). Mutations in these genes are associated with pleiotropic abnormalities in leaves along the three developmental axes (Rédei and Hirono 1964, Tsukaya and Uchimiya 1997, Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001, Iwakawa et al. 2002, Iwakawa et al. 2007), suggesting that AS1–AS2 regulates multiple genes (Takahashi et al. 2008) that might be involved in leaf formation along these axes. The AS1–AS2 complex directly represses the transcription of *BP* and *KNAT2* (Guo et al. 2008). Some of the pleiotropic abnormalities, including short leaves, of *as2* and *as1* plants have been attributed to ectopic expression of *BP*, *KNAT2* and *KNAT6* (Ikezaki et al. 2010), suggesting a role for these *KNOX* genes in the proximal–distal development of the leaf. Furthermore, transcripts levels of the *ARF3/ETT*, *KAN2* and *YAB5* genes are repressed downstream of AS1 and AS2 in shoot apices (Iwakawa et al. 2007, Takahashi et al. 2008). AS1–AS2 directly represses expression of *ARF3/ETT*, and the up-regulated *ARF3/ETT* in *as1* and *as2* is responsible for defects in both development of the adaxial domain and expansion of the leaf lamina (Iwasaki et al. 2013). These results suggest the involvement of AS1–AS2 in both adaxial development and the expansion of leaves through, at least in part, the functions of *ARF3/ETT*.

Defects in polarity of *as1* and *as2* leaves are enhanced under certain growth conditions as well as in conjunction with mutations in members of certain groups of genes (see the Introduction of Horiguchi et al. 2011b, Kojima et al. 2011, Ishibashi et al. 2012, Nakagawa et al. 2012, Xu et al. 2012), which are designated as modifiers of adaxial–abaxial patterning (Szakonyi et al. 2010, Iwasaki et al. 2013). These modifier genes include several that mediate the biogenesis of tasiR-ARF [a *trans*-acting short interfering RNA (ta-siRNA) see above]. Other relevant modifier genes belong to several different groups: those for ribosome biogenesis (Pinon et al. 2008, Yao et al. 2008, Horiguchi et al. 2011a); chromatin modification (Ueno et al. 2007, Kojima et al. 2011); genomic stability (Inagaki et al. 2009); and cell proliferation (Yuan et al. 2010, Horiguchi et al. 2011b, Ishibashi et al. 2012, Xu et al. 2012). We have previously shown that mutations of the gene encoding the histone acetyltransferase *ELONGATA3* (ELO3), a component of the Elongator complex, enhanced the polarity defects associated with *as2* (Kojima et al. 2011). The *as2 elo3* double mutant produces filamentous leaves with abaxialized epidermis. We have further reported a mutation, designated *enhancer*

of *asymmetric leaves2* and *asymmetric leaves1* (*eal*), that is associated with efficient generation of abaxialized filamentous leaves on the *as2* or *as1* background (Ishibashi et al. 2012). *EAL* encodes BOBBER1 (BOB1) (Jurkuta et al. 2009, Perez et al. 2009), an Arabidopsis ortholog of eukaryotic NudC domain proteins. Transcript levels of *ARF3/ETT*, *ARF4*, *KAN2*, *YAB5* and all four class 1 *KNOX* genes are markedly elevated in shoot apices of *as2 eal* and *as2 elo3* mutants. While these observations do suggest genetic interactions between *AS1* and *AS2* and each of these modifier genes, our understanding of the regulation mechanism for the expression of polarity-related effectors by *AS1*–*AS2* is still limited. Pathways of regulation by modifiers for the establishment of leaf polarity and cell proliferation to produce flat and symmetric leaves are largely unknown.

In the present study, we have carried out clustering analysis by KB-FuzzyART with a new gene list, Gene-list-2, by which we here report that *AS1*–*AS2* plays roles in the repression of the *Isopentenyltransferase* (*IPT*) gene for cytokinin biosynthesis and that of the *Kip-related protein* (*KRP*) genes for inhibitors of cyclin-dependent kinases (*CDKs*) through the functions of *ARF3/ETT* and *ARF4*. In addition, our results suggest that KB-FuzzyART has high potential to extract candidate genes from among thousands of genes by using gene expression data of mutant strains.

Results

***ARF3/ETT* is a common target of modifier genes, *BOB1/EAL* and *ELO3/EAST1*, in the *as2* background**

As mentioned in the Introduction, mutations of *BOB1/EAL* and *ELO3/EAST1* act as modifiers of *as1* and *as2* to enhance both the adaxial defect to generate filamentous leaves with an abaxialized surface, and the expression levels of many genes including *ARF3/ETT* increased in *as2 bob1/eal* and *as2 elo3* double mutants (Kojima et al. 2011, Ishibashi et al. 2012). In addition, introduction of the *ett* mutation into the *as2-1 eal-1* double mutant partially suppressed its phenotype of filamentous leaves (Ishibashi et al. 2012), indicating that the increased level of *ARF3/ETT* transcripts is responsible for the adaxial defect. To confirm this indication further, we examined the effects of the *ARF4* gene mutation, which is functionally redundant with *ARF3/ETT* (Pekker et al. 2005, Iwasaki et al. 2013), in the leaf phenotype of *as2-1 eal-1 ett-13*. As shown in Fig. 1H and Table 1, the *as2-1 eal-1 ett-13 arf4-1* quadruple mutant plants generated symmetrically expanded leaves and no filamentous or trumpet-shaped leaves: phenotypes were more clearly rescued in the quadruple mutant than in the *as2-1 eal-1 ett-13* triple mutant.

We subsequently ascertained the effect of the *ett-13* mutation on filamentous leaf formation in the *as2-1 elo3-27/east1-1* double mutant, because the transcript level of *ARF3/ETT* was also increased in the double mutant (Kojima et al. 2011). As shown in Fig. 1K and Table 1, 70% of the *as2-1 elo3-27 ett-13*

triple mutants exhibited expanded leaves. As shown in Fig. 1L and Table 1, the *as2-1 elo3-27 ett-13 arf4-1* quadruple mutant plants generated expanded leaves and no filamentous or trumpet-shaped leaves: the phenotypes were more clearly rescued in the quadruple mutant than in the *as2-1 elo3-27 ett-13* triple mutant. Thus, the mutations of *ARF3/ETT* and *ARF4* clearly rescued filamentous leaves generated by *as2-1 elo3-27*, although the partner in double mutations was different from *eal-1*. The results of experiments with *eal-1* and *elo3-27* suggest that the repression of *ARF3/ETT* and *ARF4* is generally critical for establishment of the adaxial domain during leaf development.

Meta-analysis to extract candidate downstream genes of *AS1*, *AS2* and *ARF3/4*

We hypothesized that the population of up-regulated genes in *as2-1 eal-1* and *as2-1 elo3-27* might include candidate effector genes more closely related to leaf abnormality, because *ARF3/ETT* and *ARF4* appear to encode transcription factors. Since levels of transcripts of abaxial determinant genes, such as *ARF3/ETT* and *ARF4*, are increased in *as2-1 eal-1* and *as2-1 elo3-27* double mutants (Kojima et al. 2011, Ishibashi et al. 2012), we can expect to extract efficiently candidate effector genes downstream of *AS1*, *AS2*, *ARF3/ETT* and *ARF4* by clustering analysis of microarray data using these double mutants.

We have already examined the gene expression profiles of Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1*, in which *AS2* was ectopically expressed (see the Materials and Methods) (Takahashi et al. 2008), by microarray analysis, and clustered genes according to their expression patterns using KB-FuzzyART, a clustering algorithm for the analysis of gene expression profiles (Takahashi et al. 2008). We re-calculated data sets of these plants by improved KB-FuzzyART described below. We named these data Dataset-A (Fig. 2A). In the present study, we examined the gene expression profiles of both *as2-1 eal-1* and *as2-1 elo3-27* double mutants by microarray analysis, and clustered identified genes according to their expression patterns by KB-FuzzyART. We named these data Dataset-B (Fig. 2A). We then searched for candidate effector genes downstream of *AS1*, *AS2*, *ARF3/ETT* and *ARF4* by meta-analysis using Dataset-A and Dataset-B (Fig. 2A).

Clustering analysis by improved KB-FuzzyART using Gene-list-2

In the previous report, we constructed and used Gene-list-1 as knowledge required for KB-FuzzyART. Since Gene-list-1 did not include some genes related to development and cell proliferation, we added 103 genes to update Gene-list-1 and renamed it Gene-list-2 (Table 2, Supplementary Table S1) (see the Materials and Methods). We examined the gene expression profiles of Col-0, *as2-1 eal-1* and *as2-1 elo3-27* by microarray analysis, and clustered genes according to their expression patterns using KB-FuzzyART. As shown in Fig. 2B, we used data sets of Col-0, *as2-1 eal-1* and *as2-1 elo3-27* (Dataset-B). The 425

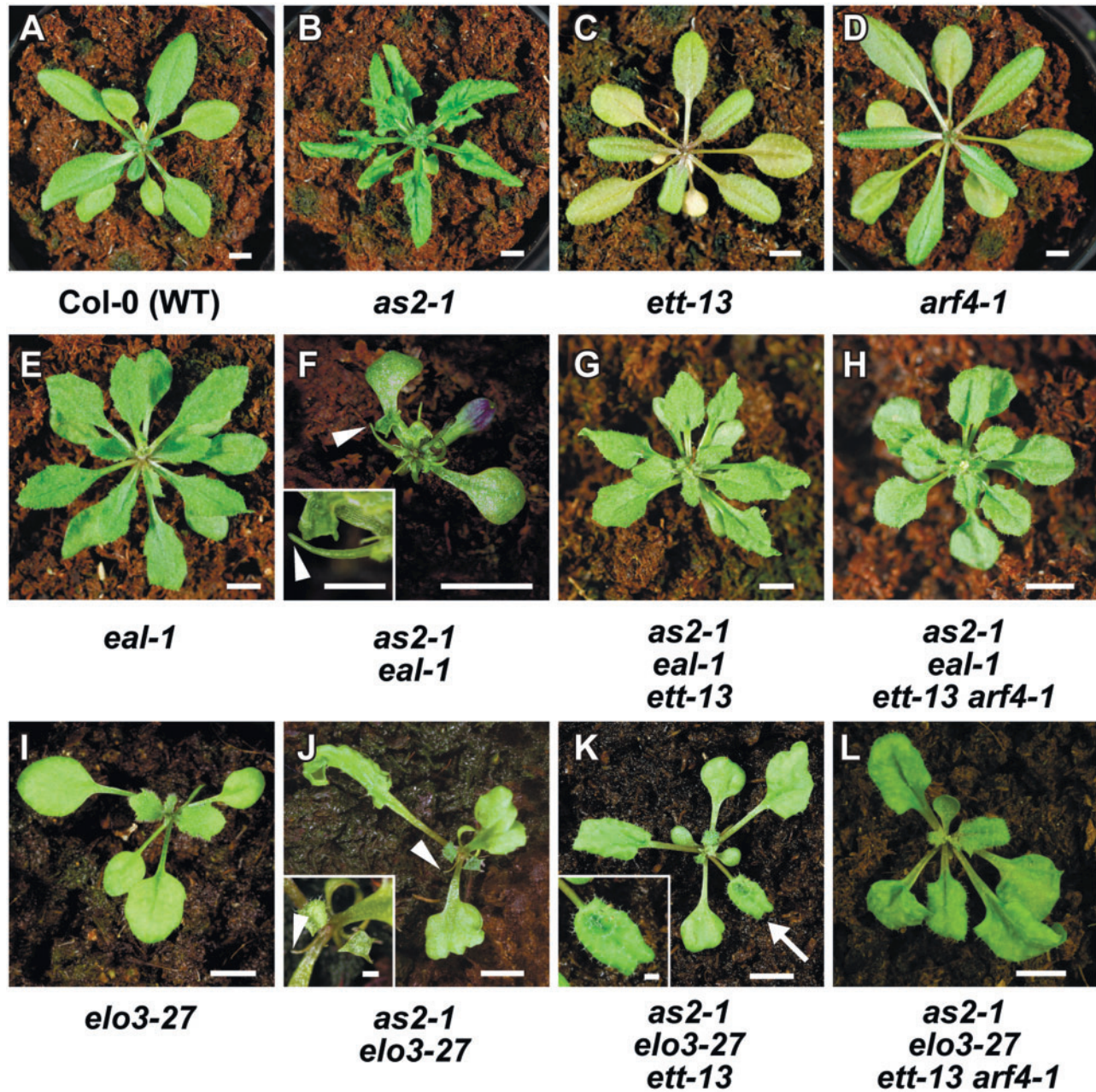


Fig. 1 The *ett* and *arf4* mutations efficiently suppressed the abaxial–adaxial defect phenotype of *as2-1 eal-1* and *as2-1 elo3-27* leaves. Gross morphology of (A) Col-0, (B) *as2-1*, (C) *ett-13*, (D) *arf4-1*, (E) *eal-1*, (F) *as2-1 eal-1*, (G) *as2-1 eal-1 ett-13*, (H) *as2-1 eal-1 ett-13 arf4-1*, (I) *elo3-27*, (J) *as2-1 elo3-27*, (K) *as2-1 elo3-27 ett-13* and (L) *as2-1 elo3-27 ett-13 arf4-1* plants is shown. Scale bars: 5 mm. Plants shown in A–H were photographed 28 d (A–H) and 21 d (I–L) after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively. Higher magnification views of filamentous leaves and trumpet-shaped leaves are shown in F, J and K. Scale bars: 1 mm in higher magnification views.

genes in Gene-list-2 were filtered and excluded 299 genes that have undetectable calls (Abs.), no-change calls (N.C.) or no probes on the ATH1 chip against the wild type in these plants. The expression data sets of the 126 filtered genes were processed by KB-FuzzyART and classified into 14 clusters, designated as clusters B1–B14 (Fig. 2B). Then 22,746 probes on the GeneChip were filtered and 17,123 genes were excluded. The

remaining 5,623 genes were assigned either as members of each cluster or as outliers. The patterns of gene expression levels in the outliers did not match those in any clusters. The outliers comprised 199 of the 5,623 genes (Table 3). Profiles of the constructed clusters and assignment of genes for Dataset-B are shown in Table 3 and Fig. 3A, B. To search for common genes downstream of *EAL* and *ELO3* on the *as2-1 eal-1* and *as2-1*

Table 1 Frequencies of plants with filamentous and trumpet-shaped leaves

Genotype	No. of plants examined	Filamentous leaves	Trumpet-shaped leaves
Col-0	64	0 (0%)	0 (0%)
<i>as2-1</i>	91	0 (0%)	0 (0%)
<i>eal-1</i>	132	0 (0%)	0 (0%)
<i>as2-1 eal-1</i>	111	84 (76%)	16 (14%)
<i>as2-1 eal-1 ett-13</i>	45	15 (33%)	8 (18%)
<i>as2-1 eal-1 ett-13 arf4-1</i>	44	0 (0%)	0 (0%)
<i>elo3-27</i>	49	0 (0%)	0 (0%)
<i>as2-1 elo3-27</i>	44	44 (100%)	35 (80%)
<i>as2-1 elo3-27 ett-13</i>	44	13 (30%)	11 (25%)
<i>as2-1 elo3-27 ett-13 arf4-1</i>	5	0 (0%)	0 (0%)

Frequency is defined by the ratio of the number of plants with more than one filamentous or trumpet-shaped leaf to the total number of plants examined. The percentages of plants with filamentous or trumpet-shaped leaves are indicated in parentheses. Plants were grown at 22°C.

elo3-27 background, we focused on genes in the clusters B1, B6 and B13, in which transcript levels of the genes were increased in both *as2-1 eal-1* and *as2-1 elo3-27* double mutants (Table 3, Fig. 3A, B). The cluster B1 contained 140 genes including *ARF4*. The cluster B6 contained 93 genes including *BP/KNAT1*, *YAB5* and *KAN2*. The cluster B13 contained 63 genes including *ARF3/ETT* (Fig. 3, Table 3, Supplementary Table S2).

We re-calculated data sets of Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1* (Dataset-A) by KB-FuzzyART using Gene-list-2. The 425 genes in Gene-list-2 were filtered and the remaining 114 genes were processed by KB-FuzzyART and classified into 18 clusters, designated as clusters A1–A18. Then 22,746 probes on the GeneChip were filtered and the remaining 4,759 genes were assigned as members of each cluster or as outliers. The outliers comprised 62 of the 4,759 genes (Supplementary Table S3). Profiles of the constructed clusters and assignment of genes for Dataset-A are shown in Supplementary Table S3 and Fig. S1A, B.

It was previously reported that AS1 and AS2 form a repressor complex that binds directly to the 5' upstream regions of the *KNOX* loci *BP/KNAT1* (Guo et al. 2008). We have also found that the AS1–AS2 complex binds to the 5' upstream regions of the *ARF3/ETT* locus and represses its expression (Iwasaki et al. 2013). Therefore, AS1–AS2 is assumed to function as a transcriptional repressor and we focused on cluster A3, which included *ARF3/ETT* and *BP/KNAT1*. The expression levels of genes in cluster A3 were up-regulated in the *as1-1* and *as2-1* plants, and down-regulated in the *AS2-eoe/as2-1* plants (Supplementary Table S3). Cluster A3 contains 38 genes that include the *BP/KNAT1*, *KNAT6*, *ARF3/ETT* and *YAB5* genes (Supplementary Table S4). Expression levels of the genes included in cluster A6 were decreased in *as1-1* and *as2-1*, but not changed in *AS2-eoe/as2-1* (Supplementary Table S3), and contained 319 genes including *KAN2* and *ARF4* (Supplementary Table S4, Fig. S1A, B). Therefore, we

focused on genes classified into cluster A6 in addition to cluster A3 in Dataset-A (Fig. 2A).

Search for candidate genes downstream of *ARF3/ETT* and *ARF4* by using KB-FuzzyART and meta-analysis

We combined the 357 genes in clusters A3 and A6 of Dataset-A and the 296 genes in clusters B1, B6 and B13 of Dataset-B, and extracted 57 genes (Fig. 2A, Table 4). These 57 genes are common downstream genes among AS1, AS2, *BOB/EAL* and *ELO3*. In addition, the 57 genes include candidates of genes regulated by *ARF3/ETT* and *ARF4*. We found that only *KRP2* and *KRP5* among the 57 genes have been known as genes that were involved in cell division, the transcript levels of which were commonly increased in *as1*, *as2* and these double mutants. Since we focused on the cell division-related genes that were downstream of AS1 and AS2, we further confirmed the transcript levels of the *KRP2* and *KRP5* genes in the double mutants by quantitative real-time RT–PCR (qRT–PCR). As shown in Fig. 4A, transcript levels of these genes increased by 2- to 4-fold in *as2-1 eal-1*. Fig. 4B shows that levels of the *KRP2* and *KRP5* gene transcripts increased by 1.5-fold in the *as2-1 elo3-27* mutant. Our results suggested that these two genes are negatively controlled downstream of AS2, *EAL1* and *ELO3*.

We further examined effects of the *ett-13* and *arf4-1* mutations on increased transcript levels of these genes in the *as2-1 eal-1* mutant. As shown in Fig. 4C, transcripts levels of *KRP2* and *KRP5* in the *as2-1 eal-1 att-13 arf4-1* quadruple mutant decreased to levels close to those of both the wild-type plant and the *att-13 arf4-1* mutant. These results suggest that *KRP2* and *KRP5* were positively regulated downstream of *ARF3/ETT* and *ARF4* in the *as2-1 eal-1* mutant.

Since we have shown that shoot regeneration from leaf sections in *as1* and *as2* and the frequencies of shoot regeneration from leaf sections of the *as1-1* and *as2-1* mutants were restored by introduction of an *ett-13 arf4-1* double mutation to that of the wild-type leaf sections, we speculated that some genes related to cytokinin synthesis and/or signaling might be downstream of AS1–AS2–*ARF3/4*. We did not find genes related to cytokinin, however, in 57 common candidate downstream genes of both the clusters A3 and A6 in Dataset-A and the clusters B1, B6 and B13 in Dataset-B. Therefore, we searched for genes related to cytokinin synthesis and (or) signaling in clusters B1, B6 and B13 and found the *IPT3* gene in cluster B13. The *IPT* genes encode adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *A. thaliana* (Kakimoto 2001, Takei et al. 2001, Sakakibara et al. 2005). We further confirmed the transcript levels of *IPT3* in the double mutants by qRT–PCR. As shown in Fig. 4A and B, transcript levels of *IPT3* increased by 3-fold in *as2-1 eal-1* and by 1.5-fold in *as2-1 elo3-27*. We also examined the effects of the *ett-13* and *arf4-1* mutations on increased transcript levels of *IPT3* in the *as2-1 eal-1* mutant. Fig. 4C shows that transcript levels of *IPT3* in the *as2-1 eal-1 att-13 arf4-1* quadruple mutant decreased. These results

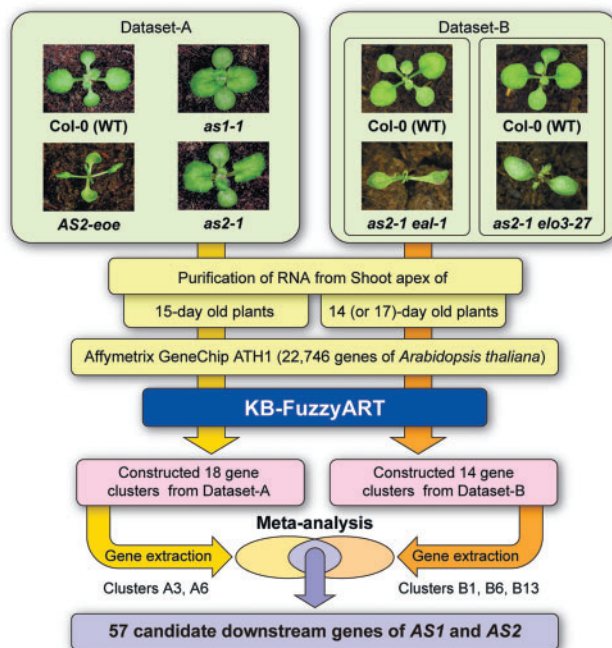
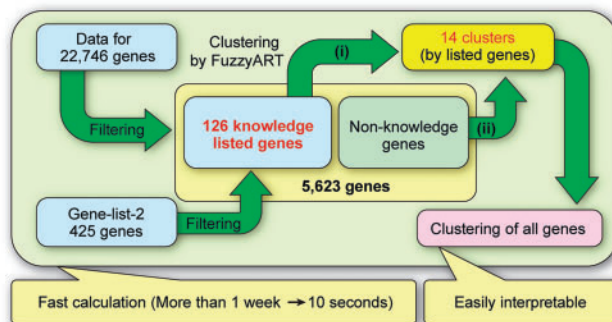
A Calculation of gene expression data**B** Scheme of clustering analysis of Dataset-B by KB-FuzzyART

Fig. 2 Gene expression analysis was conducted by the pre-processing procedure and clustering analysis by KB-FuzzyART. (A) RNA purification was conducted from the shoot apices of 15-day-old plants for Dataset-A (e.g. Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1* mutants) and of 14- or 17-day-old plants for Dataset-B (e.g. Col-0, *as2-1 eal-1* and *as2-1 elo3-27* mutants). Gene expression data were obtained by using Affymetrix GeneChip ATH1. Each array data set comprised 22,746 *A. thaliana* genes (probe sets). KB-FuzzyART analysis was conducted for two data sets, and gene clusters were constructed. Furthermore, meta-analysis (integration of several analyses) was applied to clustering results for extraction of candidate downstream genes of AS1 and AS2. (B) Clustering analysis of gene expression data (Dataset-B) was conducted by KB-FuzzyART. Each array data set and the 425 genes listed in Gene-list-2 were filtered by a pre-processing procedure (see the Materials and Methods). (i) First step: clustering of listed genes (knowledge-based listed genes) (e.g. cell fate determination genes, cell division-related genes, plant hormone-related genes). (ii) Second step: clustering of all genes was conducted by assignment to the 14 clusters constructed for Dataset-B. On the basis of this procedure, clustering by KB-FuzzyART provides the advantages of fast calculation and easy interpretation.

Table 2 Categories and numbers of genes in Gene-list-2

Category	Subcategory	No. of genes
Cell fate determination-related genes	Meristem-related genes	17
	Adaxial determinants	6
	Abaxial determinants	11
	Organ differentiation-related genes	19
	Small RNA biogenesis-related genes	24
	AS2/LOB family	41
	TCP family	17
Cell division related genes	Chromatin-related genes	8
	Cell division-related genes	61
Plant hormone-related genes	TIR1 gene and its homologs	4
	AUX/IAA family	29
	ARF family	21
	GH3 family	20
	SAUR family	69
	IPT genes	9
	AHK genes	4
	ARR family	24
	PIN genes	8
	ACS family	12
	AHP family	6
	YUCCA family	5
Unclassified genes	LSH family	10
Total		425

suggest that *IPT3* was a positively regulated gene downstream of *ARF3/ETT* and *ARF4* in the *as2-1 eal-1* mutant. Although we further examined transcript levels of *IPT5* and *IPT7*, we observed no changes of transcript levels in either the *as2-1 eal-1* or the *as2-1 elo3-27* mutant (data not shown).

AS1–AS2 acts as the repressor of multiple downstream genes in independent pathways

The *BP/KNAT1*, *ARF3/ETT* and *YAB5* genes encode transcription factors, the target genes of which have yet to be identified. We here examined whether AS1 and AS2 might repress the expression of these genes in independent pathways, or whether some of them might control others downstream of AS1 and AS2. To test these possibilities, we investigated the effects of mutations in *BP/KNAT1*, *KNAT2*, *KNAT6*, *ARF3/ETT*, *ARF4* and *YAB5* on the transcript levels of *BP/KNAT1*, *YAB5* and *ARF3/ETT* on the *as1* or *as2* background by qRT–PCR (Fig. 5). As shown in Fig. 5A, transcript levels of *BP/KNAT1* and *YAB5* were higher in the *as2-1 ett-13 arf4-1* mutant than in the *ett-13 arf4-1* mutant. These results indicate that AS1 and AS2 repress *BP/KNAT1* and *YAB5* independently from the *ARF3/ETT* and *ARF4* functions.

We next measured levels of *BP/KNAT1* and *ARF3/ETT* transcripts in the *yab5-1*, *as1-1 yab5-1* and *as2-1 yab5-1* mutants. The levels of *BP/KNAT1* and *ARF3/ETT* transcripts were higher in the *as1-1 yab5-1* and *as2-1 yab5-1* mutants than in the *yab5-1* mutant (Fig. 5B). These results indicate that repression of *BP/KNAT1* and *ARF3/ETT* by AS1 and AS2 is independent of *YAB5* functions.

Table 3 The constructed clusters and assignment of genes for Dataset-B

Cluster number	Cluster member	Pattern	
		<i>as2-1 eal-1</i>	<i>as2-1 elo3-27</i>
B1	140	+	+
B2	459	+	0
B3	1,173	–	–
B4	55	–	+
B5	603	0	+
B6	93	+	+
B7	552	–	0
B8	1,195	+	0
B9	695	0	–
B10	172	–	–
B11	43	+	–
B12	129	–	–
B13	63	+	+
B14	52	+	–
Outlier	199		
Overall	5,623		

+, – and 0 indicate up-regulation, down-regulation and no change, respectively.

Finally, we measured levels of *ARF3/ETT* and *YAB5* transcripts in the *bp-1 knat2-3 knat6-2* and *as2-1 bp-1 knat2-3 knat6-2* mutants. Transcript levels of *ARF3/ETT* and *YAB5* were higher in *as2-1 bp-1 knat2-3 knat6-2* than in *bp-1 knat2-3 knat6-2* (Fig. 5C). These results indicate that *AS1* and *AS2* repress *ARF3/ETT* and *YAB5* in a manner independent from the functions of *BP/KNAT1*, *KNAT2* and *KNAT6*.

Taken together, the results suggest that *AS1–AS2* represses transcript levels of *BP/KNAT1*, *ARF3/ETT* and *YAB5* genes in pathways independently of each other (Fig. 6).

Discussion

The *AS1–AS2–ETT* pathway controls gene networks for stabilization of the adaxial–abaxial patterning of leaves

In the present study, we have shown that the phenotypes of filamentous leaves with adaxial defects that were generated by *as2 bob1-3/eal-1* and *as2 elo3-27* were rescued by introduction of the *ett arf4* double mutation to generate flat symmetric leaves (Fig. 1). We have previously shown that the phenotype of filamentous leaves of *as1* and *as2* plants treated with 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation, is completely suppressed by the introduction of *ett-13 arf4-1* double mutations (Iwasaki *et al.* 2013). These results suggest that elevated expression of *ARF3/ETT* was responsible for the defects in adaxial development and the lack of lateral expansion. Therefore, repressive control of *ARF3/ETT* by *AS1–AS2* should be critical for stabilization of the normal development of leaves with proper adaxial–abaxial polarity and left–right

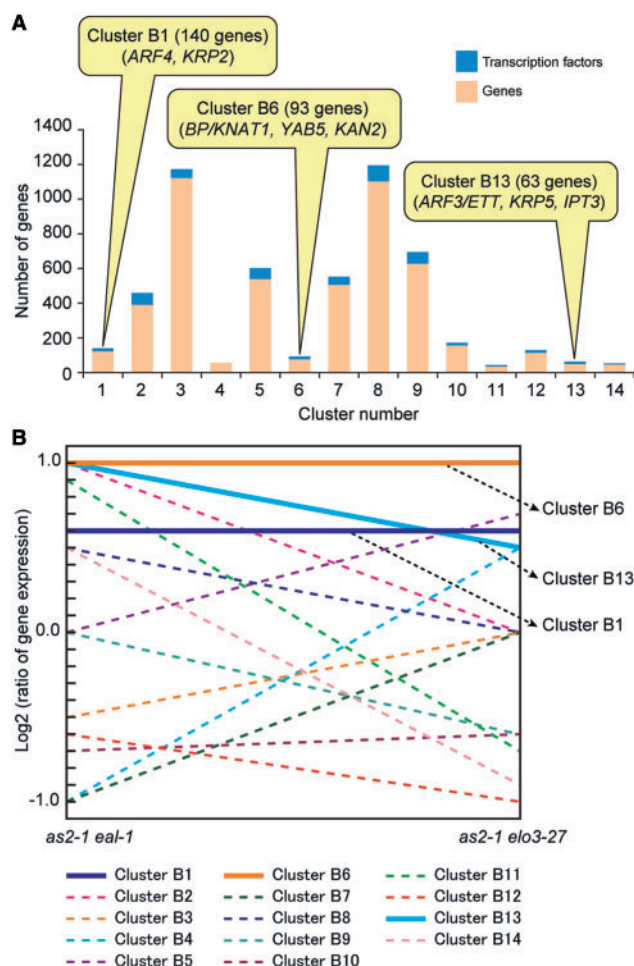


Fig. 3 Gene members were assigned by KB-FuzzyART clustering for Dataset-B. (A) Bar graph of genes as distributed within the 14 clusters of 5,623 genes. *ARF4* and *KRP2* were assigned to cluster B1. *BP/KNAT1*, *YAB5* and *KAN2* were assigned to cluster B6. *ARF3/ETT*, *KRP5* and *IPT3* were assigned to cluster B13. The blue portion indicates the number of transcription factors. (B) Clustering analysis by KB-FuzzyART. Levels of gene expression in comparison with those of Col-0 (WT) are shown. Clusters B1, B6 and B13 contained genes having elevated transcription levels in both *as2-1 eal-1* and *as2-1 elo3-27*.

symmetry. It might be possible that filamentous leaves generated in many other kinds of modifier mutants (see Introduction of Kojima *et al.* 2011) in the *as1* or *as2* genetic background might have resulted from elevated expression of both the *ARF3/ETT* and *ARF4* genes. In consideration of these possibilities, we treated *as2-1 ett-13 arf4-1* triple mutant plants with a specific inhibitor of histone deacetylase (HDAC), trichostatin A (TSA), since *as2-1* treated with TSA generates abaxialized filamentous leaves (Ueno *et al.* 2007). The phenotype of filamentous leaves in *as2-1* treated with TSA was effectively decreased in the *ett-13 arf4-1* mutant background (data not shown). Expression of *ARF3/ETT* is also regulated by several distinct mechanisms at the levels of transcription and translation (Nishimura *et al.* 2005, Ueno *et al.* 2007, Inagaki *et al.* 2009,

Table 4 The genes extracted commonly by using Dataset-A and Dataset-B

AGI code	Cluster numbers assigned by using Dataset-B	Gene symbol	Subcategory name in Gene-list-2	Cluster numbers assigned by using Dataset-A
AT1G07350	B1		–	A6
AT1G63020	B1	<i>NRPD1A</i>	–	A6
AT1G64710	B1		–	A6
AT1G76890	B1	<i>GT2</i>	–	A6
AT1G78670	B1	<i>ATGGH3</i>	–	A6
AT1G78970	B1	<i>LUP1</i>	–	A6
AT1G80530	B1		–	A6
AT2G26300	B1	<i>GP ALPHA 1</i>	–	A6
AT2G29530	B1		–	A6
AT2G39130	B1		–	A6
AT2G46590	B1	<i>DAG2</i>	–	A6
AT3G50440	B1	<i>MES10</i>	–	A6
AT3G50630	B1	<i>KRP2/ICK2</i>	Cell division-related genes	A6
AT5G01870	B1		–	A3
AT5G02030	B1	<i>BLR/RPL</i>	Meristem-related genes	A6
AT5G47180	B1		–	A3
AT5G53460	B1	<i>GLT1</i>	–	A6
AT5G57710	B1		–	A6
AT5G60450	B1	<i>ARF4</i>	Abaxial determinants	A6
AT5G61420	B1	<i>MYB28</i>	–	A6
AT1G28580	B6		–	A6
AT1G32240	B6	<i>KAN2</i>	Abaxial determinants	A6
AT1G33790	B6		–	A6
AT1G77760	B6	<i>NIA1</i>	–	A6
AT2G01670	B6	<i>atnudt17</i>	–	A6
AT2G22660	B6		–	A6
AT2G26580	B6	<i>YAB5</i>	Abaxial determinants	A3
AT2G29660	B6		–	A6
AT2G43510	B6	<i>ATTI1</i>	–	A6
AT3G09390	B6	<i>MT2A</i>	–	A6
AT3G23290	B6	<i>LSH4</i>	LSH family	A6
AT3G52800	B6		–	A6
AT4G02380	B6	<i>SAG21</i>	–	A6
AT4G08150	B6	<i>BP/KNAT1</i>	Meristem-related genes	A3
AT4G16447	B6		–	A6
AT4G34710	B6	<i>ADC2</i>	–	A6
AT4G38210	B6	<i>ATEXPA20</i>	–	A6
AT4G38620	B6	<i>MYB4</i>	–	A6
AT5G14090	B6		–	A6
AT5G39850	B6		–	A6
AT5G43580	B6		–	A6
AT5G64110	B6		–	A6
AT1G05710	B13		–	A6
AT2G05380	B13	<i>GRP35</i>	–	A6
AT2G33860	B13	<i>ARF3/ETT</i>	Abaxial determinants	A3
AT2G34650	B13	<i>PID</i>	–	A6
AT2G38870	B13		–	A6

(continued)

Table 4 Continued

AGI code	Cluster numbers assigned by using Dataset-B	Gene symbol	Subcategory name in Gene-list-2	Cluster numbers assigned by using Dataset-A
AT3G02500	B13		–	A3
AT3G13790	B13	<i>ATBFRUCT1</i>	–	A6
AT3G22550	B13		–	A6
AT3G23050	B13	<i>AXR2/IAA7</i>	AUX/IAA family	A6
AT3G24810	B13	<i>KRP5/ICK3</i>	Cell division-related genes	A6
AT4G00720	B13	<i>ATSK32</i>	–	A6
AT4G32150	B13	<i>ATVAMP711</i>	–	A6
AT4G37410	B13	<i>CYP81F4</i>	–	A6
AT5G40540	B13		–	A6
AT5G65310	B13	<i>ATHB5</i>	–	A6

–, the gene was not included as a member of Gene-list-2.

Ng et al. 2009, Iwasaki et al. 2013). In addition, mRNAs of *ARF3/ETT* and *ARF4* are both targeted by a ta-siRNA called tasiR-ARF (Allen et al. 2005, Williams et al. 2005, Fahlgren et al. 2006, Hunter et al. 2006, Nogueira et al. 2006, Nogueira et al. 2007, Chitwood et al. 2009, Schwab et al. 2009). The presence of these multiple processes for *ARF3/ETT* repression implies that the negative control of *ARF3/ETT* (probably also *ARF4*) as well as class 1 *KNOX* genes is essential for the normal formation of vegetative and floral lateral organs in *A. thaliana* (Tabata et al. 2010).

AS1–AS2 regulates *KRP* and *IPT* genes through *ARF3/ETT* and *ARF4* for leaf development

The present study has also shown that expression of *KRP2*, *KRP5* and *IPT3* is positively controlled downstream of *ARF3/ETT*, at least on the *as2* and modifier backgrounds, suggesting that *ARF3/ETT* has abilities to elevate the endogenous level of cytokinin and to repress cell cycle progression (Fig. 6).

What is the developmental relevance of the repressive controls of these three genes by AS1–AS2? We postulate that repression of *KRP2*, *KRP5* and *IPT3* by AS1–AS2 through *ARF3/ETT* functions might be related to the stabilization of leaf development (Fig. 6).

KRP2 and *KRP5* of *A. thaliana* encode cyclin-dependent kinase inhibitors (CKIs) (De Veylder et al. 2001). Leaf formation might also require that cell proliferation that might be achieved by the properly repressive control of *CKI* genes by AS1–AS2 (Fig. 6). Some *CKI* genes are predicted to be involved in endoreduplication in *A. thaliana*, which eventually leads to cell cycle arrest (Ormenese et al. 2004). *KRP2*, for example, is expressed in tissues in which endoreduplication occurs, and its overexpression (or ectopic expression) results in an increase in the ploidy level (Verkest et al. 2005). Our previous observation that the rate of endoreduplication is stimulated during leaf formation in *as2-1 eal-1* double mutants (Ishibashi et al. 2012) is consistent with the results in the present study. There might be a correlation between the progression of endoreduplication and cell

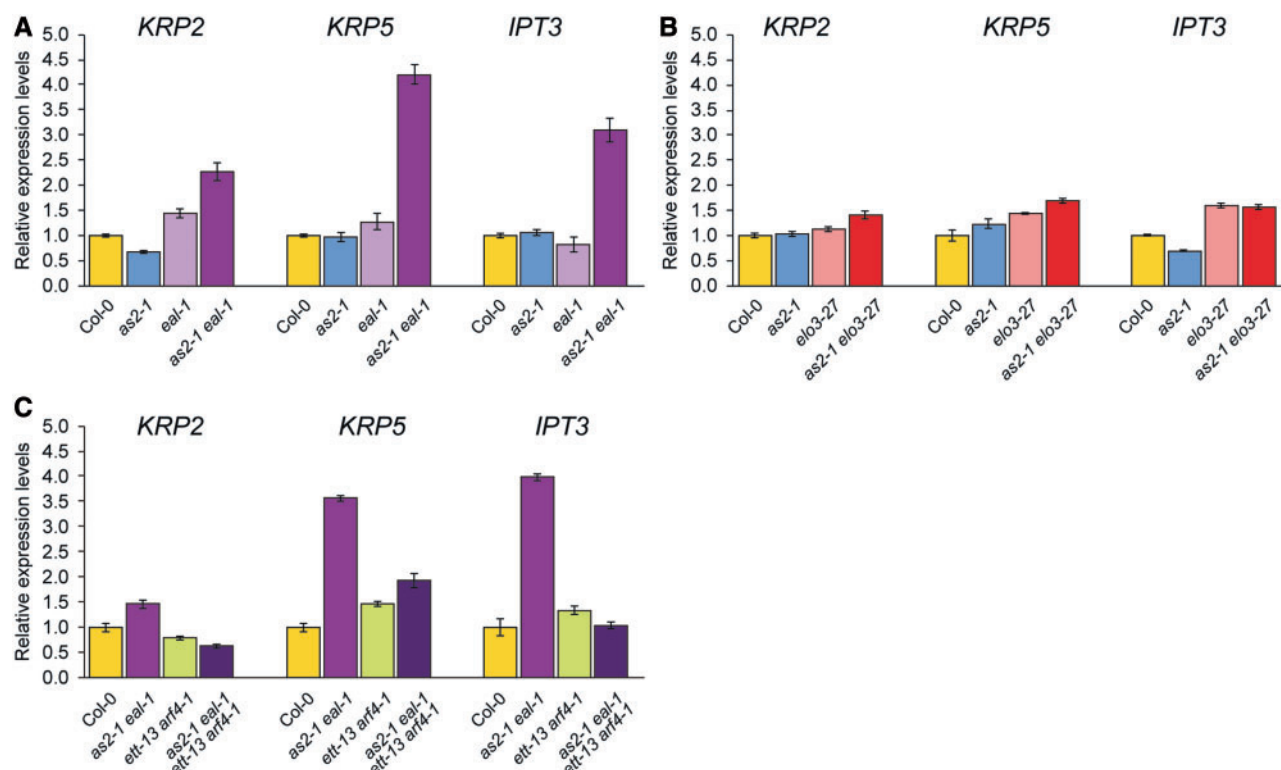


Fig. 4 Levels of transcripts of *KRP2*, *KRP5* and *IPT3* genes in shoot apices of various mutants measured by quantitative real-time RT-PCR (qRT-PCR). Transcript levels are shown for (A) Col-0, *as2-1*, *eal-1* and *as2-1 eal-1* mutants; (B) Col-0, *as2-1*, *elo3-27* and *as2-1 elo3-27* mutants; and (C) Col-0, *as2-1 eal-1*, *ett-13 arf4-1* and *as2-1 eal-1 ett-13 arf4-1* mutants. Total RNA of plants in (A) and (C) was extracted from short apices of seedlings 17 d after sowing. Total RNA of plants in (B) was extracted from short apices of seedlings 14 d after sowing. Each value was normalized by reference to the level of *ACTIN2* transcripts. The values from Col-0 plants were set arbitrarily at 1.0. Bars indicate the SD among more than three biological replicates.

differentiation in plants (Joubès and Chevalier 2000, De Veylder et al. 2002, Inzé and De Veylder 2006). *KRP2* is expressed in mitotically dividing cells (Ormenese et al. 2004). Therefore, AS1–AS2 might be involved in the modulation of a balance between cell cycle progression and cell differentiation by the proper repression of expression of these CKI genes.

CKIs, which interact with CDKs to inhibit their kinase activities, act as key regulators of cell cycle progression (Sherr and Roberts 1999). Typical examples are p21, p27 and p57 in animal cells (Toyoshima and Hunter 1994, Lee et al. 1995). A role for the CKIs in cell differentiation was reported during muscle development and maintenance of adult hematopoietic stem cells (Zhang et al. 1999, Matsumoto et al. 2011); however, little is known about how the cell cycle machinery communicates with developmental processes in both plants and animals. Understanding of molecular networks that involve CKIs must shed light on the coordination between cell proliferation and the developmental processes in multicellular organisms.

The endogenous level of cytokinin might increase around the SAM in *as1* and *as2* mutants because of the ectopic expression of *IPT3*, which might affect developmental states of cells in the leaf primordia of *as1* and *as2* mutants. In fact, the regeneration potential of shoots in leaves of the *as1* and *as2* mutants

increases during *in vitro* culturing (Semiarti et al. 2001). This phenotype is consistent with the above prediction of an increase in the cytokinin level in these mutant leaves and has recently been shown to contribute to the elevated expression of *ARF3/ETT* in *as1* and *as2* (Iwasaki et al. 2013). Thus, an unnecessary increase of cytokinin should disturb the stabilization of the developmental status of leaves. For further understanding of developmental roles of the AS1–AS2–ETT–IPT3 pathway in cytokinin synthesis, endogenous levels of cytokinin in *as1* and *as2* must be examined by further experiments.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Col-0 (CS1092), *as1-1* (CS3374) and *as2-1* (CS3117) were obtained from the Arabidopsis Biological Resource Center (ABRC). We outcrossed *as2-1* with Col-0 three times and *as1-1* with Col-0 once, and used the progeny for our experiments (Semiarti et al. 2001). For analysis of phenotypes, seeds were sown in soil or on plates of MS (Murashige and Skoog) medium. After 2 or 3 d at 4°C in darkness, plants were transferred to a daily regime of 8 h of darkness

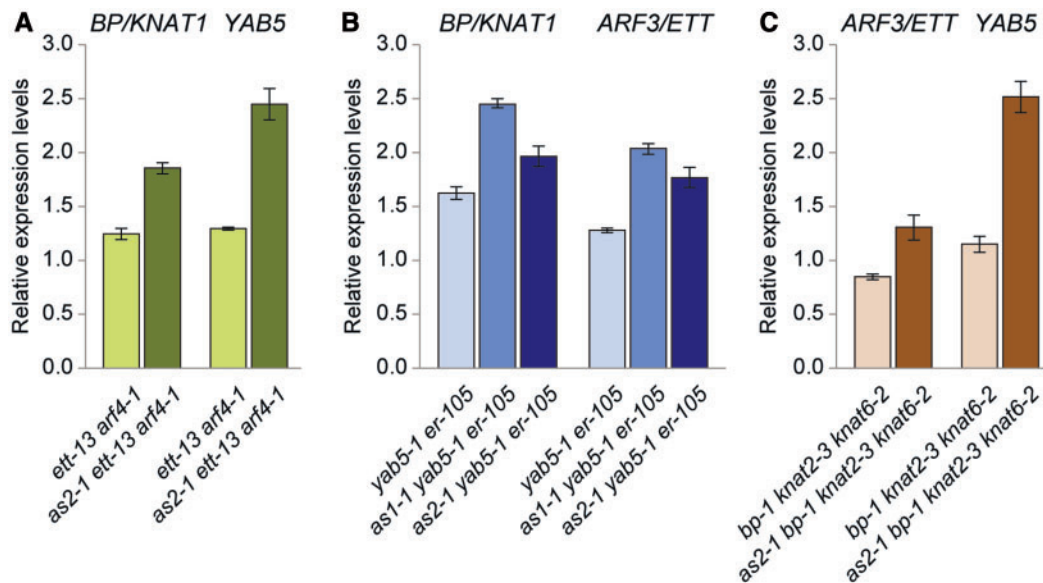


Fig. 5 Transcript levels of *BP/KNAT1*, *YAB5* and *ARF3/ETT* genes in the shoot apices of various 15-day-old mutants as measured by quantitative real-time RT–PCR (qRT–PCR). Shown are the transcript levels of (A) *BP/KNAT1* and *YAB5* in *ett-13 arf4-1*, *as1-1 ett-13 arf4-1* and *as2-1 ett-13 arf4-1* mutants; (B) *BP/KNAT1* and *ARF3/ETT* in the *yab5-1 er-105*, *as1-1 yab5-1 er-105* and *as2-1 yab5-1 er-105* mutants; and (C) *ARF3/ETT* and *YAB5* in the *bp-1 knat2-3 knat6-2*, *as1-1 bp-1 knat2-3 knat6-2* and *as2-1 bp-1 knat2-3 knat6-2* mutants. Each value was normalized by reference to the level of *ACTIN2* transcripts. The values from Col-0 plants were set arbitrarily at 1.0. Bars indicate the SD among more than three biological replicates.

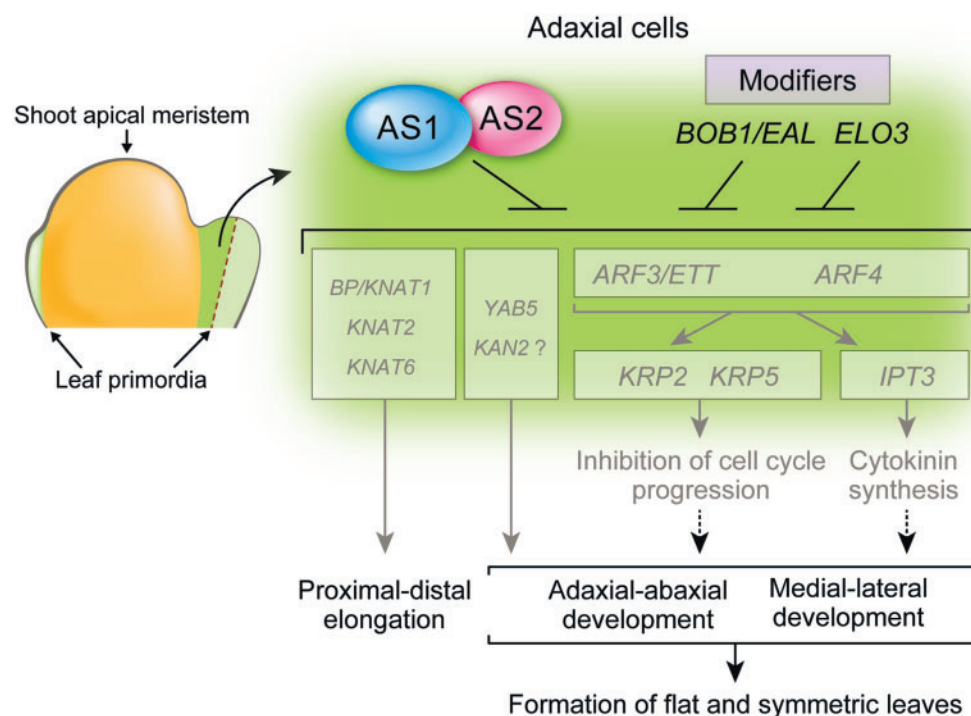


Fig. 6 Model for regulation of *ARF3/ETT* and *ARF4* by the AS1–AS2 complex in the early stage of leaf primordia in *A. thaliana*. Temporal regulation of the gene expression for establishment of leaf adaxial–abaxial polarity is shown in *A. thaliana*. The leaf primordium in the early stage has begun to grow outward and then acquired polarity in the radial dimension (Lynn et al. 1999). In the leaf primordium AS1–AS2 represses class 1 *KNOX* and abaxial determinant genes, together with *EAL* and *ELO3*. AS1–AS2 and modifiers regulate *KRP* and *IPT* genes through *ARF3/ETT* and *ARF4*.

and 16 h of white light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C , as described previously (Semiarti et al. 2001). Ages of plants are given in terms of numbers of days after sowing. The *east1-1/elo3-27* and *bob1-3/eal-1* mutants were previously described (Kojima et al. 2011, Ishibashi et al. 2012). The seeds of *ett-13* and *arf4-1* were kindly provided by Dr. Yuval Eshed (Weizmann Institute of Science, Israel). The *ett-13* allele contains a loss-of-function mutation with a T-DNA insertion at 18 bp downstream of the translation start site of *ETT* (Pekker et al. 2005). The *arf4-1* allele is also a loss-of-function mutation with a *Ds* insertion (Parinov et al. 1999). Both *ett-13* and *arf4-1* are presumptive null, or close to null, alleles. The *yab5-1* mutant, which has a stop codon mutation in the YABBY domain, was obtained from the Arabidopsis TILLING Project (Till et al. 2003). This plant has not only *yab5-1* but also *er-105* mutations. These two mutations could not be segregated by backcrossing three times because of the short distance between *YAB5* and *ER* (<100 kbp). The *bp-1*, *kmat2-3* and *kmat6-2* mutants were previously described (Ikezaki et al. 2010). The transgenic plant *AS2-eoe/as2-1*, in which *AS2* was ectopically expressed under the control of the *AS1* promoter (pAS1:AS2; designated *AS2-eoe*), was also previously described (Iwakawa et al. 2002, Iwakawa et al. 2007).

Microarray analysis

Arrays of Dataset-A were described previously (Takahashi et al. 2008). For Dataset-B, shoot apices of Arabidopsis plants were harvested 14 or 17 d after sowing, and immediately frozen in liquid nitrogen and stored at -80°C . Total RNA extracted from each sample of shoot apices containing leaf primordia and young leaves was reverse transcribed, yielding double-stranded cDNA, which was transcribed in vitro in the presence of biotin-labeled nucleotides using a GeneChip 3'IVT Express Kit (Affymetrix Inc.), and purified. Labeled amplified antisense RNA was fragmented and hybridized to Affymetrix ATH1 GeneChip arrays for 16 h at 45°C according to Affymetrix protocols. Arrays were then washed on an Affymetrix Fluidics Station 450 and measured for fluorescence intensity with an Affymetrix GeneChip Scanner 3000 7G. The microarray data of Dataset-A presented in this paper are available from the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44028>) under the accession number GSE44028.

Data processing

Raw data processing was performed by using Affymetrix Gene Chip Operating Software (GCOS) (Version 1.4.0.036). We used two sets of gene expression data, Dataset-A obtained from strains Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1*, and Dataset-B obtained from strains Col-0, *as2-1 eal-1* and *as2-1 elo3-27*. Each set of array data comprised 22,746 plant genes (probe sets). Initially, we calculated the expression signals for all strains and the $\log_2(\text{ratio})$ for three strains (*as1-1*, *as2-1* and *AS2-eoe/as2-1*) and two strains (*as2-1 eal-1* and *as2-1 elo3-27*) against Col-0 by GCOS. In this experiment, we excluded 64 controls and

2,177 genes subject to cross-hybridization, according to NetAffx Annotation (www.affymetrix.com). Furthermore, for each group set, we excluded those genes for which all strain data sets showed an absent call (i.e. the detection call determined by GCOS based on the *P*-value of the one-sided Wilcoxon signed-rank test; an absent call means $P \geq 0.065$, which is the default threshold in GCOS), because it indicates that the expression signal was undetectable. We also excluded those genes for which all strain data sets showed a no change call [i.e. no change call means $(1-0.006) \geq P \geq 0.006$ for one-sided Wilcoxon signed-rank test], because no change indicates that the expression signal is almost equal to that of Col-0. Thus, 4,759 and 5,623 genes were selected for Dataset-A and Dataset-B, respectively. Among these data, the $\log_2(\text{ratio})$ values that were >2-fold or <0.5-fold were rounded to 2-fold and 0.5-fold, respectively; the $\log_2(\text{ratio})$ values with no change were rounded to 0, to avoid category proliferation in clustering.

Gene-list-2 for *Arabidopsis thaliana*

In the present study, we used KB-FuzzyART. This is a powerful algorithm for clustering of biological data (Takahashi et al. 2008); however, KB-FuzzyART is a knowledge-based system that requires some knowledge data. Therefore, we constructed Gene-list-1 for *A. thaliana* genes in a previous study (Takahashi et al. 2008). We added 103 genes (e.g. chromatin-related genes, ACS family, LSH family and *PIN* genes) to update Gene-list-1, and renamed it Gene-list-2, which comprises 425 genes (Table 2). Further detailed information of Gene-list-2 is shown in Supplementary Table S1.

The clustering by KB-FuzzyART

We applied KB-FuzzyART for two data sets: Dataset-A (Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1*) and Dataset-B (Col-0, *as2-1 eal-1* and *as2-1 elo3-27*). First, for KB-FuzzyART, FuzzyART was applied to any knowledge, such as the 114 and 126 genes obtained from Gene-list-2 for each data set, respectively. The KB-FuzzyART parameters (i.e. vigilance parameter and learning rate) were optimized by using an optimal clustering index (OCI) (Takahashi et al. 2003), and then 18 and 14 clusters were constructed, respectively, for each data set in the present study. Weight vectors (representative patterns for each cluster) for the 18 and 14 clusters were constructed by FuzzyART, and the learning rate was set to 0, to fix the constructed weight vectors. FuzzyART with 18 or 14 fixed weight vectors was then applied to the 4,759 or 5,623 filtered genes for each data set. All of the 4,759 or 5,623 genes were assigned to each cluster or outliers (62 or 199 genes) for each data set. The outliers represent genes with patterns that did not match patterns from Gene-list-2.

Real-time RT-PCR

Leaves and shoot apices of wild-type and mutant plants were harvested at 14 or 17 d after sowing, and immediately frozen in liquid nitrogen and stored at -80°C . Total RNA was isolated by

using the RNeasy Plant Mini Kit (Qiagen). For the analysis of RNA levels by real-time PCR, we prepared 5 µg of total RNA to convert to cDNA as described by Iwakawa et al. (2007). PCR was performed in the presence of the double-stranded DNA-specific dye Power SYBR Green (Applied Biosystems) with primers as described (Iwakawa et al. 2007, Takahashi et al. 2010). Amplification was monitored in real time by using the Applied Biosystems StepOnePlus Real-Time PCR system, according to the supplier's recommendations. The mean value of three technical replicates was normalized by the value of ACTIN2 transcripts. Primers used are listed in [Supplementary Table S5](#).

Supplementary data

[Supplementary data](#) are available at PCP online.

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